thetic grouping derived from vitamin A or retinene would yield no materials not known to be present in the eye.

CONCLUSIONS

The modulator analogues which have been obtained fulfil nearly all the requirements of the colour receptors whose presence is implied by Granit's work, e.g. during fading processes, the maxima fall at different rates, suggesting the presence, not of one product with several bands, but of several products with one maximum to each. The conditions under which these modulator analogues have been produced are obviously unphysiological.

If the problem posed by Granit's modulators is put in the form of a query whether or not the vitamin A-retinene oxidation-reduction system could possibly account for the phenomena observed, the answer is clearly in the affirmative. Which catalysts, old or new, must be invoked before the modulator analogues can be obtained under less obviously unphysiological conditions is a matter for further research.

SUMMARY

1. The absorption spectra of vitamin A and of retinene$_1$ in concentrated sulphuric, phosphoric and hydrochloric acids have been studied. Sharp absorption bands characteristic of unstable ionized molecules were obtained.

2. The results show that vitamin A and retinene$_1$ can give rise *in vitro* to materials simulating the photopic modulators of Granit.

3. The hypothesis that the system vitamin A-retinene$_1$ is important in photopic as well as scotopic vision is strengthened.

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Studies in Vitamin A

11. REACTIONS OF RETINENE$_1$ WITH AMINO COMPOUNDS

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Rhodopsin or visual purple is a conjugated protein obtained from dark-adapted retinae of many species. The prosthetic group is responsible for the colour ($\lambda_{max.}$ 500 mμ.) and photosensitivity, and is derived from vitamin A or its aldehyde retinene$_1$. No explanation has so far been advanced to account satisfactorily for the displacement of $\lambda_{max.}$ from either 328 mμ. (vitamin A) or 370–390 mμ. (retinene$_1$) to 500 mμ.

The decomposition product of rhodopsin known as indicator yellow shows $\lambda_{max.}$ 440 mμ. in acid solution and 365 mμ. in alkaline solution. Neither the pH sensitivity nor the 440 mμ. maximum has been properly accounted for.

The interaction of purified retinene$_1$ with amino compounds throws some light on these problems. A preliminary account of the work (Ball, Collins, Morton & Stubbs, 1948) has appeared, and the present paper carries the study a stage further.

EXPERIMENTAL

General procedure (*a*). A solution of crystalline retinene$_1$ in ethanol (about 10^{-6} M) was prepared so that the optical density (*E*) at 380–390 mμ. was 1·5–2·5, for a 1 cm. layer. To the solution (2 ml.) was added an aqueous solution of amino compound (2 ml.). In some cases the latter solution was nearly saturated, and in all cases the concentration exceeded 10^{-3} M. To the mixture, 0·1 N aqueous NaOH (2 ml.) was added, and the final mixture was left to stand at room temperature for 15–30 min. The absorption spectrum of the solution was then measured. Next, conc. HCl (1 drop) was added and the absorption spectrum redetermined, using a compensating cell containing water. The Beckman spectrophotometer was used throughout. In general, the alkaline solutions showed $\lambda_{max.}$ 360–370 mμ. and the acidified solutions $\lambda_{max.}$ 435–460 mμ.

General procedure (*b*). A number of amino compounds in which the NH_2 group is attached directly to the benzene ring produce a reddish colour with retinene$_1$ in the presence of acid. Pretreatment with alkali is not necessary, and $\lambda_{max.}$ appears at 490–535 mμ. depending on the nature of the amino compound.

Conditions of interaction of retinene$_1$ and amino compounds. The effect of varying the molecular ratio amino compound/retinene$_1$ has been investigated in the case of β-alanine.

RESULTS

A variety of compounds containing amino groups has been tried by procedure (*a*). Urea, formamide and dimethylamine significantly failed to shift the

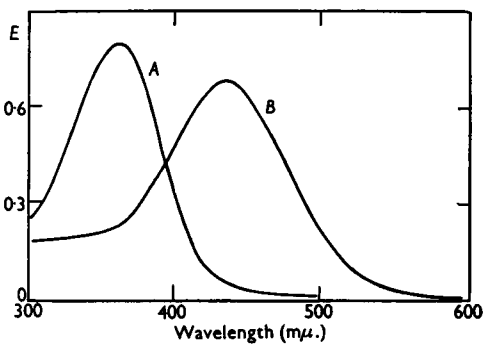


Fig. 1. Absorption spectrum of retinene$_1$ plus methylamine (in excess). *A*, after standing (10 min.) in alkaline medium; *B*, subsequently acidified.

retinene$_1$ absorption spectrum. Simple amines and aliphatic amino-acids, as well as tyrosine and tryptophan, reacted slowly with retinene$_1$ in alkaline solution giving a displacement of $\lambda_{max.}$ from 380–390 to 360–370 mμ. On acidification a new band $\lambda_{max.}$ 435–460 mμ. appeared (Figs. 1 and 2).

The effect lacks specificity, since a variety of proteins show it, and the only difficulty is that instead of an orange yellow solution, an orange precipitate is sometimes formed (Table 1).

Aniline and various compounds related to it produce on the other hand a colour in acid solution immediately on mixing, no treatment with alkali

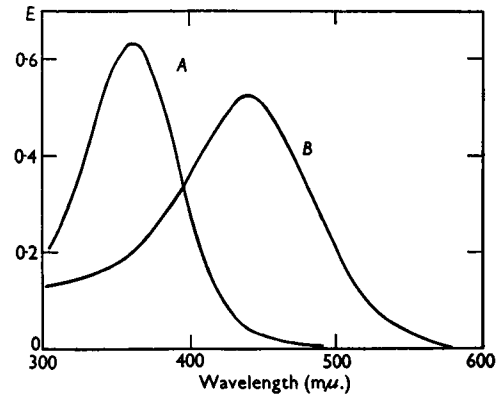


Fig. 2. Absorption spectrum of retinene$_1$ plus β-alanine (in excess). *A*, after standing (10 min.) in alkaline medium; *B*, after subsequent acidification. Retinene concentration $1·3 \times 10^{-5}$ M; 1 cm. cell.

Table 1. *Wavelengths of maximum absorption of mixtures of retinene$_1$ and amino compounds in aqueous ethanol*

(The mixtures were first made alkaline and left to stand and then acidified. The amino compound was in considerable excess over retinene$_1$.)

Amino compound	$\lambda_{max.}$ in alkali (mμ.)	$\lambda_{max.}$ in acid (mμ.)
Methylamine	360–365	435
Dimethylamine	385–390	385–390 (no change)
Benzylamine	365	445
Urea	385–300	385–390 (no change)
Formamide	385–390	385–390 (no change)
Glycine	372	440–445
β-Alanine	365	440
Serine	370	435–440
Isoleucine	370	450–455
Tyrosine	365	445
Tryptophan	360–365	445
Glutamic acid	375	435–440
Lysine	365	440–445
Arginine	365–370	460
Egg albumin	370	450
Peptone	365	440
Edestin	380	455
Trypsin	Solution not clear	440–450
Gelatin	360	440
Casein	360	Orange yellow precipitate
Zein	360–365	Orange yellow precipitate

being necessary. (Fig. 3, aniline, Fig. 4, *p-amino*-benzoic acid.) This colour is due to a chromophore with $\lambda_{max.}$ 490–535 mμ. (Table 2).

Table 2. *Wavelengths of maximum absorption of mixtures of retinene$_1$ and amino compounds, which result in visible colour in acid solutions without preliminary treatment with alkali.*

Amino compound	$\lambda_{max.}$ in acid solution (mμ.)
Aniline	490–500
Methylaniline	490
Benzylaniline	490
Dimethylaniline	No colour produced
1-Naphthylamine	500
2-Naphthylamine	515
p-Toluidine	500
o-Aminophenol	505
p-Aminobenzoic acid	530
Anthranilic acid	520
p-Aminobenzenesulphonamide	520
Diphenylamine	535

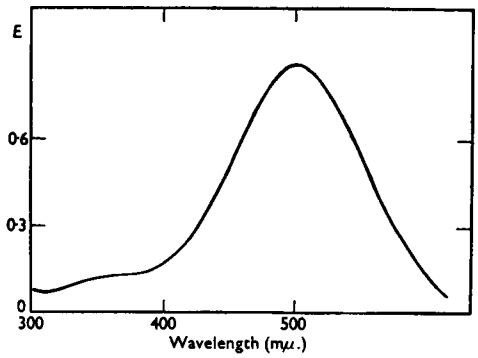


Fig. 3. Absorption spectrum of retinene$_1$ plus aniline (in excess) in acid solution; retinene concentration $1{\cdot}58 \times 10^{-5}$ M; 1 cm. cell.

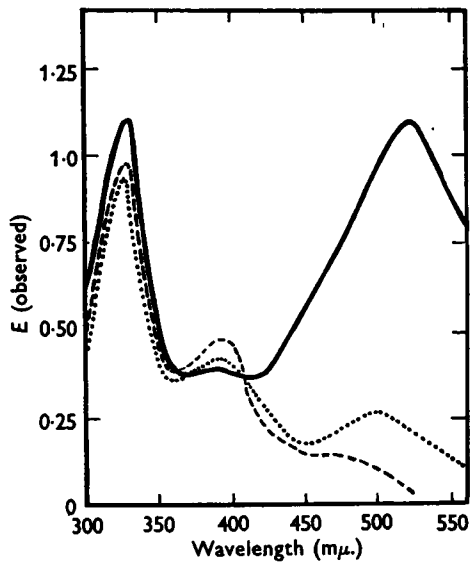


Fig. 4. Absorption spectrum of retinene$_1$ plus *p*-aminobenzoic acid, acidified; ——, solution examined at once, ..., after 6 hr. exposure to diffused daylight, - - - - -, after 10 hr. exposure to daylight.

In procedure (*a*) it is necessary to have the amino compound in excess over the retinene$_1$ if the change in $\lambda_{max.}$ from 380–390 to 360–370 mμ. in alkali is to be effected quickly (e.g. in 10 min.). The molecular ratio of, for example, β-alanine/retinene needs to be about 200, but a smaller excess will show the displacement in 48 hr. (Table 3). Even in quite alkaline media (0·033 M-NaOH) there is no shift in $\lambda_{max.}$ when β-alanine and retinene$_1$ are mixed in equimolecular proportions. With excess of β-alanine the reaction occurs readily in 0·033 M-NaOH, but very slowly in neutral solution (measured pH 6·98), a small shift only being obtained after 48 hr.

Table 3. *Interaction of retinene$_1$ and β-alanine in* 0·033 M-NaOH *and ethanol*

(The mixture was left to stand and then acidified with one drop conc. HCl; retinene, $1{\cdot}3 \times 10^{-5}$ M; β-alanine in excess.)

Molar ratio β-alanine/retinene	Alkaline solution $\lambda_{max.}$ (mμ.)	Acidified solution $\lambda_{max.}$ (mμ.)	Time, NaOH solution was left to stand
0·5	385	385	48 hr.
1	375	385	48 hr.
30	370	395	48 hr.
60	390	395	10 min.
60	365	407	48 hr.
120	370	410–415	10 min.
180	370	425	10 min.
240	365	430	10 min.
480	365	440	10 min.
600	365	440	10 min.

Retinene$_1$ (33 mg.) and β-alanine (5 mg.) were dissolved in ethanol (2 ml.), 0·1 N ethanolic NaOH (2 ml.) added, the mixture allowed to stand for several hours in the dark and then acidified with concentrated hydrochloric acid. The solution became dark red ($\lambda_{max.}$ 440–450 mμ., using a small portion diluted with ethanol). If one drop of the red solution was diluted with water, the colour disappeared and $\lambda_{max.}$ occurred at about 390 mμ. The solute obtained by removal of solvent *in vacuo* from the red solution was insoluble in light petroleum, but soluble in chloroform ($\lambda_{max.}$ 465 mμ.). The red solution showed no spectroscopic evidence of free retinene in appreciable proportion, although the retinene$_1$ and β-alanine were used in the molecular ratio 2 : 1.

Those amino compounds which result in $\lambda_{max.}$ of about 500 mμ. permit an approximately quantitative study of colour intensity (*E* at 490–500 mμ.) in relation to concentration of reactants and combining proportions. This is possible for the 500 mμ. chromogen because retinene$_1$ has no appreciable absorption at that wavelength, whereas the overlapping of the 440 mμ. absorption and retinene$_1$ absorption is a serious complication. Benzylaniline, which gives $\lambda_{max.}$ 490 mμ. although it has only one available hydrogen, was used for the stoicheiometric test. Table 4 illustrates the results. The assumption

Table 4. *Interaction of retinene$_1$ and benzylaniline in acid solution*

$$K = \frac{[\text{resultant}]}{[\text{retinene}]\,[\text{amine}]}$$

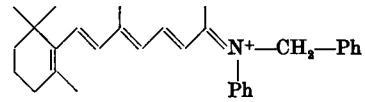
Retinene concentration ($M \times 10^{-5}$)	Benzylaniline concentration ($M \times 10^{-5}$)	E490mμ. (corr.)	K
1·59	1·59	0·139	$1·85 \times 10^4$
1·59	3·18	0·246	$1·95 \times 10^4$
1·59	7·95	0·392	$1·68 \times 10^4$
1·59	15·9	0·516	$1·69 \times 10^4$
1·59	Large excess	0·723	—

that one molecule of retinene reacts with two molecules of benzylaniline does not lead to an equilibrium constant, but a reasonably good constant is obtained on the basis of one molecule of retinene reacting with one molecule of benzylaniline.

DISCUSSION

To some extent the large excess of amino compounds needed in these experiments reflects the fact that very high dilution is needed for the spectrophotometry. The evidence, however, points to the interaction of two molecules of retinene$_1$ with one molecule of methylamine, glycine, etc., since one molecule of retinene$_1$ with one molecule of aliphatic amine could scarcely give the indicator yellow type of spectrum. This implies that conjugated proteins resembling and including indicator yellow itself possess two retinene$_1$ molecules attached to the same amino nitrogen atom. The molecular extinction coefficients of the methylamine derivative are about 53,000 and 45,600 in alkaline and acid media respectively (calculated/mole of retinene$_1$). The β-alanine derivative shows 'ϵ' values of about 49,000 and 40,000. As the ϵ values for vitamin A and retinene$_1$ are respectively 50,000 and about 40,000, the chromophoric change is confined to a displacement of wavelength.

The structural possibilities for the interaction of retinene$_1$ and *N*-benzylaniline are limited and the structure shown here is the most plausible one. There



(acid form)

are serious objections against the assumption that the results of Table 1 suggest that rhodopsin contains a nitrogen atom attached both to an aromatic ring and a retinene type of molecule—especially since that would imply a different linkage in indicator yellow from that in rhodopsin. Indeed it is not necessarily true that rhodopsin is a retinene, rather than a vitamin A derivative, although indicator yellow is certainly derived structurally from retinene$_1$. These, and other points concerning indicator yellow obtained from retinae, will be discussed in a later paper.

SUMMARY

1. Retinene$_1$, left to stand for a short time in alkaline solution with excess of many amino compounds and proteins, exhibits the absorption spectrum of alkaline indicator yellow, and on acidification shows that of acid indicator yellow.

2. Retinene$_1$, treated in acid solution with aniline and similar compounds, gives a red solution with $\lambda_{max.}$ about 500 mμ. This shows that retinene can give rise to a rhodopsin-like chromophore although in a different way from rhodopsin.

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Ball, S., Collins, F. D., Morton, R. A. & Stubbs, A. L. (1948). *Nature, Lond.*, **161**, 424.